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Steroid Hormone Quantification in Nails with LC-MS/MS Using $^{13}C_3$-labeled Surrogate Analytes

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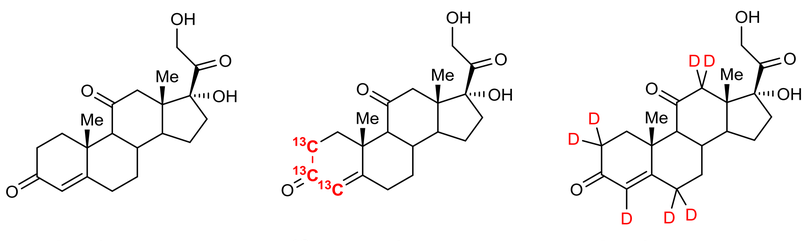
1. Introduction

The analysis of endogenous steroid hormones in hair is increasingly used in the area of stress and health-related research for the long-term determination of those biomarkers. If hair is not available, nails can be used as an alternative matrix for the retrospective evaluation of substances. The aim of the project was to develop a sensitive LC-MS/MS method for the quantification of steroid hormones in human nails. A new approach with the use of $^{13}C_3$-labeled surrogate analytes was applied for the quantification of endogenous compounds in authentic matrix.



Cortisone	$^{13}C_3$-Cortisone	D_7-Cortisone
360.4 g/mol	363.4 g/mol	367.4 g/mol

Fig. 1: Structures of cortisone, the surrogate analyte $^{13}C_3$-cortisone and the internal standard D_7-cortisone.

2. Sample preparation

Final sample preparation was defined as follows:

- **Preparation**
  - 10 mg nails (milled)
  - 50 µL IS (D_7-Cortison, D_9-Progesteron, D_5-DHEA)
- **MeOH Extraction**
  - MeOH (1 mL) extraction in ultrasonic bath for 2 h
  - Supernatant transferred, drying under N_2
- **LLE Extraction**
  - 1.5 mL ethyl acetate and 220 µL water added
  - Shaken for 10 min, centrifuged, frozen for 60 min
- **Concentration**
  - Transfer of supernatant, drying under N_2
  - Reconstitution in 350 µL eluent A and 150 µL MeOH

Fig. 2: 10 mg nails were mixed with 1 mL MeOH and 50 µL IS-mixture. After extraction in the ultrasonic bath for 2h, the supernatant was transferred and dried. After a liquid-liquid extraction with ethyl acetate, the upper layer was dried and reconstituted.

3. LC-MS/MS method

- LC: Prominence UFLC system (Shimadzu, Kyoto, Japan) equipped with a Phenomenex® Kinetex® XB-C_{18} (2.6 µm, 50 x 2.10 mm) column.
- Eluent: 0.2 mM NH_4F in water/methanol 97/3 v/v (A) and 0.2 mM NH_4F in water/methanol 3/97 v/v (B)
- MS: QTRAP® 6500+ linear ion trap quadrupole mass spectrometer (Sciex, Darmstadt, Germany).
- Measured in ESI positive in multiple reaction monitoring mode.
- Analyzing 12 steroid hormones plus three deuterated internal standards and five $^{13}C_3$-labeled hormones.
- Separation of all steroid hormones was achieved in a total run time of 12 min with good selectivity and sensitivity (Fig. 2)

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4. Validation

- The method was validated according to GTFCh guidelines.
- Six analytes (aldosterone, corticosterone, 11-deoxycortisol, 11-deoxycorticosterone, 17-OHP and DHT) were spiked to a nail pool where the concentration of these analytes where sufficiently low (“blank” matrix).
- $^{13}C_3$-cortisone, $^{13}C_3$-cortisol, $^{13}C_3$-androstenedione, $^{13}C_3$-testosterone, $^{13}C_3$-DHEA and $^{13}C_3$-progesterone were used as surrogate analytes and spiked to an authentic nail pool (Fig. 1).
- Linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, matrix effect, recovery and robustness were tested and showed good results.

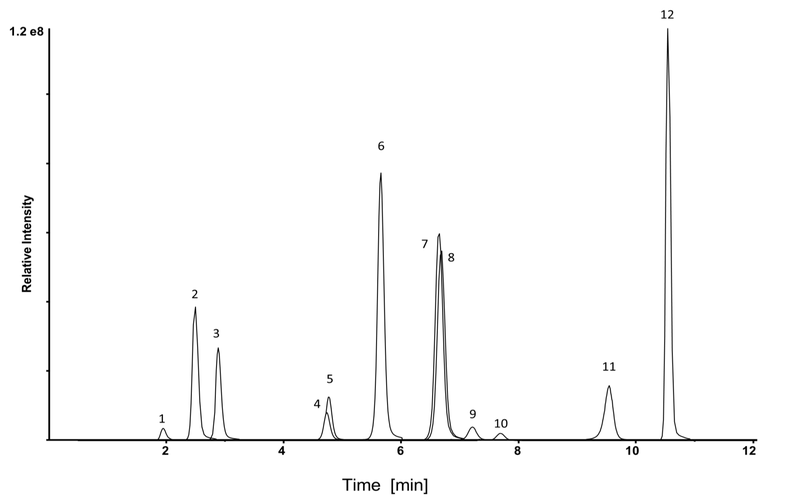

Fig. 2: Final chromatogram of the scheduled LC-MS/MS method obtained from a neat solution mix of 1 ng/µL. 1 = aldosterone, 2 = cortisone, 3 = cortisol, 4 = corticosterone, 5 = 11-deoxycortisol, 6 = androstenedione, 7 = 11-deoxycorticosterone, 8 = testosterone, 9 = DHEA, 10 = 17-OHP, 11 = DHT, 12 = progesterone, 13 = pregnenolone.

5. Application - study design

- Method was applied to nails from a study in cooperation with the Institute of Psychology from the University of Zurich.
- This sample collection happened within the assessment phase which was conducted within a larger project on the psychobiological adaptation of women to naturally occurring stress during pregnancy. The entire project was approved by the Cantonal Ethics Committee of Zurich (KEK-ZH-NR: 2012-0408).
- 31 right and left hand nails from mothers (n=12) (before and after birth) and 8 samples from infants were analyzed.

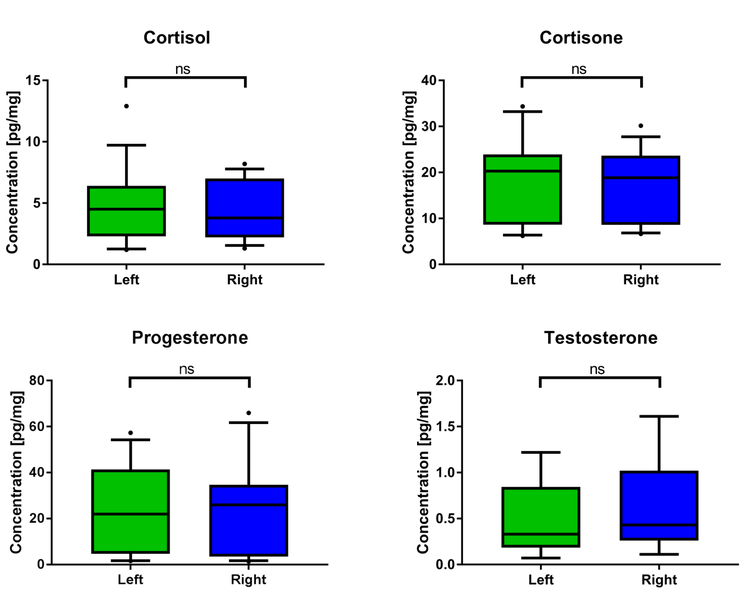

Fig. 3: The differences between left and right hand were evaluated to be not significant by a paired Wilcoxon matched-pairs signed rank test for cortisol, cortisone, progesterone and testosterone. N=15.

Conclusion

A highly selective and sensitive LC-MS/MS method for the quantification of 12 steroid hormones (aldosterone, cortisone, cortisol, corticosterone, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone, DHEA, 17-OHP, DHT, progesterone) in nails was successfully established. Using a surrogate analyte approach, the method development and validation showed that the use of $^{13}C_3$-labeled steroid hormones is a valid method in case of lack of analyte-free matrices. We showed that fingernail clippings can be used for retrospective measurement of steroid hormones in infants and adults which can be helpful in the field of hormone research.

6. Results

- Cortisol, cortisone, progesterone, testosterone, androstenedione, and 11-deoxycorticosterone were detectable in nails (Table 1).
- The comparison of steroid concentrations of cortisol, cortisone, and progesterone (which were the main steroids detected in fingernails of mothers and infants) showed no significant concentration (unpaired Mann-Whitney-Test, $p > 0.05$) difference in nails of infants compared to their mothers.
- The steroid levels in left and right hand showed no significant differences (paired Wilcoxon matched-pairs signed rank test) for cortisone, cortisol, testosterone and progesterone (Fig. 3).
- A good linear relationship was found between left and right hand for cortisol (Spearman r = 0.92, $p < 0.0001$), cortisone (Spearman r = 0.78, $p < 0.001$) and progesterone (Spearman r = 0.93, $p < 0.0001$).
- A positive linear correlation between cortisol and cortisone levels (Fig. 4) in nails was found (Spearman r = 0.89, $p < 0.0001$).

Hormone	Infants Mean [pg/mg]	Mothers Mean [pg/mg]
Cortisol	3.9, N=7	4.5, N=31
Cortisone	26.7, N=7	17.5, N=31
Progesterone	7.5, N=7	24.3, N=31
Testosterone	< LOQ	1.1, N=11
Androstenedione	< LOQ	5.7, N=13
11-Deoxycorticosterone	< LOQ	8.5, N=1

Table 1: Mean steroid hormone concentrations in nails. N shows the number of samples where the hormones could be quantified.

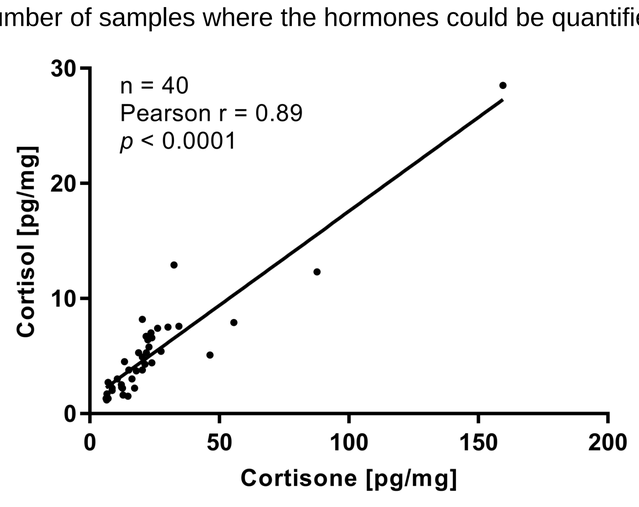

Fig. 4: Pearson correlation between cortisone and cortisol concentrations in human fingernails.

References

Voegel, C.D., La Marca-Ghaemmaghami, P., Ehlert, U., Baumgartner, M.R., Kraemer, T., Binz, T.M., Steroid Profiling in Nails Using Liquid Chromatography-Tandem Mass Spectrometry, Steroids 2018, **140**, 144-150.